

REMARKS/ARGUMENTS

This is in response to the Office Action mailed December 9, 2003.

Amendments to the Specification

On page 20 of the originally filed specification, after paragraph 69, in Table 4, row number 4, in the column titled "Dairy consumption..." the number listed should be 102.8 ± 3.6 instead of the incorrect listing of 1346 ± 113 . This was due to an obvious typographical error considering that the number in the previous column is the same, 1346 ± 113 . The correct number is in Table 4 of an article previously submitted in an information disclosure statement showing the correct number, Zemel et al., "Regulation of adiposity by dietary calcium," The FASEB Journal; Vol. 14, No. 9, pp. 1132-1138 [2000]. A copy of the article is attached for the Examiner's convenience.

Amendments to the Claims

Claims 22, 23, 24, 26 and 27 have been amended and new claims 35-77 have been added. Currently pending are independent claims 21, 25, 38 and 69, and dependent claims 22-24, 26-29 and 35-37, 39-68 and 70-77, a total of 52 claims.

Claim 24 has been amended to include the character " β " which appears to have been inadvertently left out of the term 1- β , 25, dihydroxyvitamin D due to a word processing error. Claims 22, 23, 26 and 27 have been amended to clarify explicitly the implicit binding of the antagonist, without intending to narrow the scope of the claims. New claims 35-77 have been added to particularly point out and distinctly claim the subject matter which applicants regard as the invention.

The amended claims and newly added claims cover subject matter that is supported in the originally filed specification. Support can be found in the originally filed specification, e.g., for claims 22-24, 26-27 and 39-51, see paragraphs 34, 35 and 36; for claims 35-37, see paragraph 46; for claim 38, see paragraphs 34 and 37; for claims 52, 57 and 58, see paragraph 39; for claim 56, see paragraphs 1 and 3; for claims 36, 53-55, 59-65 and 67-77, see Example 4; and for claim 66, see paragraph 66. No new matter is added.

Statutory Rejection

Claims 23 and 27 were rejected under 35 U.S.C. §112, first paragraph, for allegedly failing to comply with the enablement requirement. The Examiner contends that the claims contain subject matter which was not enablingly described in the specification. The Examiner asserts that the Applicants do not disclose how to make the claimed "chemical compounds" which are 1, 25-Dihydroxy vitamin D receptor antagonists.

The Applicant disagrees with the Examiner's rejection of non-enablement in regard to the "chemical compounds" as set forth in claims 23 and 27. The current claims are directed to methods of modulating, attenuating, or decreasing obesity in an individual comprising the administration of either (a) 1, 25-dihydroxyvitamin D (1,25-(OH)₂-D) *receptor antagonist* or (b) 1,25-(OH)₂-D *antagonist*. Examples of such antagonists are the chemical compound 1- β , 25, dihydroxyvitamin D and calcium. The methods of each of the claims are enabled and sufficiently and extensively described throughout the specification, for example at figures 1A-B, 2, 12A-F, 13A-B, 14 and 15A-B; paragraphs 32, 34, 35 and 38; and Example 4, paragraphs 89-104, all of which would enable one skilled in the art to practice the invention without undue experimentation.

The Office has the burden of establishing a lack of enablement, applying the factors articulated by the Court of Appeals for the Federal Circuit in *In re Wands*, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). These factor include:

(1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.

In this case, the Office Action has not set forth adequate evidence through clear, factor-by-factor analysis to demonstrate that one skilled in the art would find the specification nonenabling in light of its discussion and exemplification.

The Examiner in this case merely states that claims 23 and 27 are not enabled because "the Applicants do not disclose how to make the claimed "chemical compounds which are 1, 25-dihydroxyvitamin D (1,25-(OH)₂-D) receptor antagonist." The Examiner does not provide any factors, reasons or evidence that would lead one to conclude that the specification fails to teach how to make and use the claimed invention without undue experimentation. Based on the foregoing analysis, it respectfully is submitted that the Office Action has not set forth a *prima facie* case of nonenablement, and that in any event, the claims are enabled.

Claim Rejections - 35 U.S.C. §112, second paragraph

Claims 21-27 and 29 were rejected under 35 U.S.C. §112, second paragraph, for allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner contends that in "claims 21, 25 and 27, 'Receptors' is vague. What do the receptors do upon binding? Where are they located?" In addition, the Examiner contends that in "claim 25 'antagonist' is vague; what does the antagonist do?" The

Examiner also contends that in "claim 26 'antibody' is vague; antibody against what?"

Applicants traverse the rejection because those skilled in the art would understand what is claimed when the claim is read in light of the content of the present application disclosure, the teachings of the prior art and the claim interpretation that would be given by one possessing the ordinary level of skill in the pertinent art at the time the invention was made. The terms receptor, antagonist, and antibody are clear and definite and readily known to one skilled in the art.

The term *receptor* refers to a chemical group or molecule (as a protein) on the cell surface or in the cell interior that has an affinity for a specific chemical group, molecule, or virus (*see* Merriam-Webster Online dictionary at <<http://www.m-w.com>>). In this case, the receptor has an affinity to 1, 25-dihydroxyvitamin D (1,25-(OH)₂-D). The receptor of the claims is identified throughout the specification as 1,25-(OH)₂-D receptor, for example at paragraph 38, which also cites a variety of references that discuss said receptor.

The term *antagonist* refers to a chemical compound or antibody that acts as an agent of physiological antagonism, that acts within the body to reduce the physiological activity of another chemical substance, or that opposes the action of a drug or a substance occurring naturally in the body by combining with and blocking its receptor (*see* Merriam-Webster Online dictionary at <<http://www.m-w.com>>). As used in the claims it refers to either an antagonist of 1, 25-dihydroxyvitamin D (1,25-(OH)₂-D) receptor or of 1,25-(OH)₂-D.

The term *antibody* in this case is one that has an affinity for binding to and is either an antagonist of 1, 25-dihydroxyvitamin D (1,25-(OH)₂-D) receptor or of 1,25-(OH)₂-D.

These terms are sufficiently and extensively described throughout the specification, for example at figures 1A-B, 2, 12A-F, 13A-B, 14 and 15A-B; paragraphs 32, 34, 35 and 38; and

Example 4, paragraphs 89-104.

The specification, at paragraph 34, describes methods comprising decreasing the levels of calcitrophic hormones ($1,25-(\text{OH})_2\text{-D}$) in an individual by administration of a therapeutically effective amount of a $1,25-(\text{OH})_2\text{-D}$ antagonist, for example dietary calcium. Other $1,25-(\text{OH})_2\text{-D}$ antagonists include $1,25-(\text{OH})_2\text{-D}$ neutralizing antibodies; soluble $1,25-(\text{OH})_2\text{-D}$ receptor; fusion proteins comprising the $1,25-(\text{OH})_2\text{-D}$ receptor (for example soluble forms of the $1,25-(\text{OH})_2\text{-D}$ receptor fused to Ig heavy chains or moieties which preferentially target adipocytes); chemical compounds; and compounds containing calcium, such as calcium carbonate.

At paragraph 35 of the specification, the subject invention is said to provide methods comprising the administration of therapeutically effective amounts of $1,25-(\text{OH})_2\text{-D}$ receptor antagonists. Examples of such antagonists include antibodies which block the ligand binding site of the receptor; chemical compound antagonists of the receptor; and analogs, homologs, or isomers of $1,25-(\text{OH})_2\text{-D}$ which specifically bind to the $1,25-(\text{OH})_2\text{-D}$ receptor but which antagonize the function of the receptor, for example $1-\beta$, 25-dihydroxyvitamin D.

In Example 4, paragraph 102 refers to previous reports which demonstrate that the terms receptor and antagonist have well known meaning. E.g., see Bouillon, B., *et al.*, "Structure-function relationships in the vitamin D endocrine system," *Endocrine Rev.* (1995) pp. 200-57, vol. 16; and Norman, A.W., "Receptor for $1\alpha,25-(\text{OH})_2\text{-D}_3$: past, present, and future," *J. Bone Miner. Res.* (1998) pp. 1360-69, vol. 13). These references, which were cited in the specification, are submitted herewith.

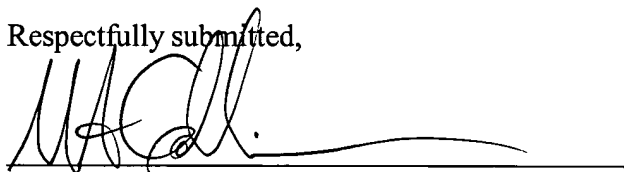
Paragraph 102 discusses the exemplary antagonist $1-\beta,25$ -dihydroxyvitamin D_3 , which blocks rapid physiological response elicited by $1\alpha,25-(\text{OH})_2\text{-D}_3$ and $1\alpha,25$ -dihydroxylumisterol $_3$

(another agonist). The meaning of the term antagonist is known in the art, e.g., Norman, A., *et al.*, "Demonstration that 1 β ,25-dihydroxyvitamin D₃ is an antagonist of the nongenomic but genomic biological response and biological profile of the three A-ring diastereomers of 1 α ,25-dihydroxyvitamin D₃", *J. Biol. Chem.* (1993) pp. 20022-30, vol. 268. This reference, which was cited in the specification, is submitted herewith.

Applicants respectfully submit that the claims are enabled, clear and definite and that their application is in condition for allowance. Should any questions remain, please contact the undersigned.

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Regulation of adiposity by dietary calcium

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ABSTRACT Recent data from this laboratory demonstrate that increasing adipocyte intracellular Ca^{2+} results in a coordinated stimulation of lipogenesis and inhibition of lipolysis. We have also noted that increasing dietary calcium of obese patients for 1 year resulted in a 4.9 kg loss of body fat ($P < 0.01$). Accordingly, we tested the possibility that calcitrophic hormones may act on adipocytes to increase Ca^{2+} and lipid metabolism by measuring the effects of $1,25\text{-(OH)}_2\text{-D}$ in primary cultures of human adipocytes, and found significant, sustained increases in intracellular Ca^{2+} and a corresponding marked inhibition of lipolysis ($\text{EC}_{50} \sim 50 \text{ pM}$; $P < 0.001$), suggesting that dietary calcium could reduce adipocyte mass by suppressing $1,25\text{-(OH)}_2\text{-D}$. To test this hypothesis, we placed transgenic mice expressing the *agouti* gene specifically in adipocytes on a low (0.4%) Ca/high fat/high sucrose diet either unsupplemented or with 25 or 50% of the protein replaced by non-fat dry milk or supplemented to 1.2% Ca with CaCO_3 for 6 wk. Weight gain and fat pad mass were reduced by 26–39% by the three high calcium diets ($P < 0.001$). The high calcium diets exerted a corresponding 51% inhibition of adipocyte fatty acid synthase expression and activity ($P < 0.002$) and stimulation of lipolysis by 3.4- to 5.2-fold ($P < 0.015$). This concept of calcium modulation of adiposity was further evaluated epidemiologically in the NHANES III data set. After controlling for energy intake, relative risk of being in the highest quartile of body fat was set to 1.00 for the lowest quartile of Ca intake and was reduced to 0.75, 0.40, and 0.16 for the second, third, and fourth quartiles, respectively, of calcium intake for women ($n = 380$; $P < 0.0009$); a similar inverse relationship was also noted in men ($n = 7114$; $P < 0.0006$). Thus, increasing dietary calcium suppresses adipocyte intracellular Ca^{2+} and thereby modulates energy metabolism and attenuates obesity risk.—Zemel, M. B., Shi, H., Greer, B., DiRienzo, D., Zemel, P. C. Regulation of adiposity by dietary calcium. *FASEB J.* 14, 1132–1138 (2000)

Key Words: human adipocytes • lipolysis • *agouti* • PTH

INTRACELLULAR Ca^{2+} PLAYS a key role in the metabolic disorders associated with obesity and insulin

resistance (1–3). Recombinant agouti protein, an obesity gene product, stimulates Ca^{2+} influx in a variety of cells (4, 5). Agouti also stimulates the expression and activity of fatty acid synthase, a key enzyme in *de novo* lipogenesis, and inhibits basal and agonist-stimulated lipolysis in human and murine adipocytes via a Ca^{2+} -dependent mechanism (6, 7). These effects can be mimicked in the absence of agouti by either receptor or voltage-mediated Ca^{2+} channel activation and inhibited by Ca^{2+} channel antagonism (6–8); agouti-induced obesity in transgenic mice was markedly attenuated by 4 wk of Ca^{2+} channel antagonism (9). Thus, increasing adipocyte intracellular Ca^{2+} appears to promote triglyceride storage in human adipocytes by exerting a coordinated control of lipogenesis and lipolysis, serving to simultaneously stimulate the former and inhibit the latter.

During the course of a previous, unrelated clinical trial investigating the antihypertensive effect of calcium in obese African-Americans, we noted that increasing daily calcium intake from ~ 400 to 1000 mg/day for 1 year resulted in a 4.9 kg reduction in body fat (Fig. 1). Although these data were inexplicable at the time, our recent data demonstrating regulation of adipocyte energy storage by intracellular Ca^{2+} lead to the proposal that increases in circulating calcitrophic hormones [$1,25\text{-(OH)}_2\text{-D}$ and/or parathyroid hormone] secondary to low calcium diets stimulate adipocyte Ca^{2+} influx and thereby increase lipid storage. If this is correct, then increasing dietary calcium should suppress calcitrophic hormones and thereby reduce adipocyte intracellular Ca^{2+} and lipid storage. The present study was conducted to address this concept.

MATERIALS AND METHODS

Isolation and culture of human adipocytes

Human subcutaneous adipose tissue was obtained from patients with no known history of metabolic disorders undergo-

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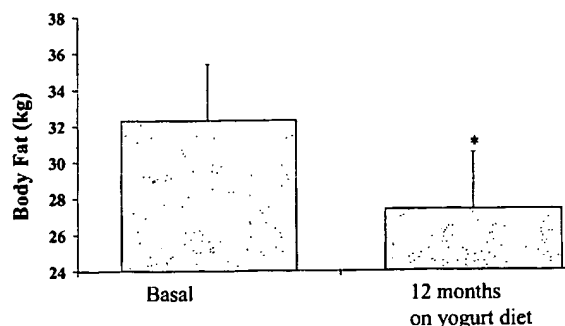


Figure 1. Effects of increasing dietary calcium for 12 months on body fat in obese African-American males. Supplemental calcium was provided in the form of two cups of yogurt/day. Calcium intake was estimated from random prospective dietary records collected 1 day each week for each subject throughout the study. Baseline dietary calcium was estimated, during a 4 wk lead-in period, to be 447 ± 126 mg/day and increased to 1029 ± 74 mg/day during the 12 months of supplementation. Body fat was assessed using bioelectrical impedance (14). Data are mean \pm standard deviation; $n=11$; * $P<0.01$.

ing abdominal plastic surgery. Adipocytes were isolated by washing, mincing, collagenase digestion, and filtration as described previously (12, 17) and cultured in Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μ g/ml), and gentamicin (50 μ g/ml). Approval for this procedure was obtained from the Institutional Review Board for Human Subjects of the University of Tennessee. Cells were cultured in suspension and maintained in a thin layer at the top of the culture media, which was changed every day. Cells were studied ~ 72 h after isolation and were serum-starved prior to study.

Animals and diets

To evaluate the role of dietary calcium in regulating adiposity *in vivo*, transgenic mice expressing *agouti* specifically in adipocytes under the control of the aP2 promoter were studied. We have previously reported a characterization of these animals. Briefly, they exhibit a normal pattern of leptin expression and activity similar to that found in humans and exhibit a human pattern (adipocyte-specific) of *agouti* expression (10). We have found these mice to be useful models for diet-induced obesity in that they are not obese on a standard AIN-93G diet, but become obese in response to hyperinsulinemia induced by either insulin administration (10) or high sucrose diets (11). Male aP2-*agouti* transgenic animals from our colony were placed at 6 wk of age on a modified AIN 93-G diet with suboptimal calcium (0.4%), sucrose as the sole carbohydrate source, and fat increased to 25% of energy with lard. They were randomized to four groups, as follows. The basal group continued this diet with no modifications; a high calcium group received the basal diet supplemented with CaCO_3 to increase dietary calcium by threefold to 1.2%; a medium dairy diet, in which 25% of the protein was replaced by non-fat dry milk and dietary calcium was increased to 1.2%; and a high dairy group in which 50% of the protein was replaced by non-fat dry milk, increasing calcium to 2.4%. Food intake and spillage was measured daily, and animals were weighed weekly. At the conclusion of the 6 wk feeding period, animals were killed by exsanguination under isoflurane anesthesia, and blood was collected via cardiac puncture

for glucose and insulin measurements. Fat pads (epididymal, perirenal, abdominal, and subscapular) were dissected, immediately weighed, frozen in liquid nitrogen, and stored at -80°C . Fatty acid synthase activity and mRNA levels were measured in abdominal fat as described below.

Core temperature

Core temperature was used as an indirect metabolic index to determine whether any reduction in efficiency of conversion of food energy to body weight was accompanied by increased thermogenesis (9). Temperature was measured via a thermocouple (Columbus Instruments, Columbus, Ohio). The probe was inserted a constant distance (1.8 cm) into the rectum of each animal. After stabilization (10 s), the temperature was recorded every 5 s for 30 s (9). All temperature measurements were made between 8:00 and 9:00 A.M.

Intracellular calcium (human adipocytes)

Intracellular Ca^{2+} was determined fluorometrically as described previously (17). Cells were washed with HEPES-buffered salt solution loaded with Fura-2-acetoxymethyl ester (10 μM) for 45 min at 37°C in the dark with continuous shaking. Cells were then rinsed three times, resuspended, and intracellular Ca^{2+} was measured using dual excitation (340 and 380 nm)/single emission (510 nm) fluorometry. After the establishment of a stable baseline, the response to $1,25\text{-(OH)}_2\text{-D}$ or parathyroid hormone (10 pM-100 nM) or their respective vehicles was determined. Digitonin (25 μM) and Tris/EGTA (100 mM, pH 8.7) were used to for calibration to calculate the final intracellular Ca^{2+} (18).

Lipolysis

Adipocytes were incubated for 4 h in the presence or absence of forskolin (1 μM), and glycerol release into the culture medium was measured (18) to assess lipolysis. Glycerol release data was normalized for cellular protein.

Fatty acid synthase activity and mRNA levels

Immediately after death, adipose tissue was isolated and fatty acid synthase activity was measured in cytosolic extracts by measuring the oxidation rate of NADPH, as described previously (6, 9, 17). Enzyme activity was protein corrected using Coomassie blue dye.

Total RNA was extracted by cesium chloride density gradient, electrophoresed, subjected to Northern blot analysis, and hybridized with a radiolabeled rat cDNA probe for fatty acid synthase using standard methods (6, 12, 17). Autoradiographs were quantitated densitometrically, and all blots were stripped and reprobed with β -actin as a loading control.

Statistical analysis (*in vitro* and animal data)

All data are expressed as mean \pm SD. Data were evaluated for statistical significance by one-way analysis of variance (ANOVA) or *t* test, depending on the number of comparisons made. All data sets with multiple comparisons were analyzed via ANOVA, followed by separation of significantly different group means via test the least significant difference using SPSS-PC (v. 8.0).

TABLE 1. Characteristics of NHANES III Study sample^a

	Women (n = 380)	Men (n = 7114)
Age (years)	28.7 ± 0.4	43.5 ± 0.44
Body mass index (kg/m ²)	25.7 ± 0.4	26.6 ± 0.11
Body fat (%)	32.7 ± 0.6	25 ± 0.2
Calcium intake (mg/day)	720 ± 52	965 ± 15
Dairy product consumption (monthly frequency)	54.7 ± 3	51.4 ± 1
Energy intake (kcal/day)	1896 ± 68	2656 ± 28
Dietary fat (g/day)	74 ± 4	102 ± 2

^a All data presented as mean ± SEM.

NHANES III analysis

To determine whether the animal observations are relevant in defining a role for dietary calcium in modulating body composition at the population level, an analysis of the National Health and Nutrition Examination Survey (NHANES III) data set was conducted. This large cross-sectional survey conducted between 1988 and 1994 followed a complex, four-stage probability sampling scheme (13) designed to represent the entire U.S. civilian noninstitutionalized population over the age of 2 months. Only adults completing all three phases of the study (interview, physical examination, and laboratory examination) were included in this data analysis; respondents were excluded from this analysis if they could not provide complete, usable body composition/anthropometric data (e.g., amputees and individuals wearing casts), used insulin, or were pregnant, recently pregnant, or currently breast-feeding. Body composition was assessed using the anthropometric and bioelectrical impedance data collected during the physical examination, with percent body fat calculated using the regression equations derived by Segal (14).

Odds ratios for percent body fat and corresponding 95% confidence intervals were estimated by multiple logistic regression analysis with a robust variance estimation method using SUDAAN (15). Point estimates for all parameters were weighted to reflect the population distribution of each; variances were calculated using SUDAAN (15) to take the complex sampling design into account, as failure to account for the weighting and design effects of a complex sample design will result in distortion of estimates and underestimation of variance. Analyses were conducted separately for men and women, and all odds ratios were adjusted for age by including age in the model as a continuous variable. Other covariates included in the model were caloric intake, race/ethnicity, and activity level. Characteristics of the study sample are shown in Tables 1 and 2.

TABLE 2. Quartiles of body fat for women and men in the NHANES III Study^a

Quartile	% Body fat	
	Women	Men
1	22.82 ± 0.45	15.47 ± 0.14
2	30.54 ± 0.17	22.83 ± 0.06
3	34.85 ± 0.18	27.55 ± 0.06
4	42.49 ± 0.44	34.08 ± 0.11

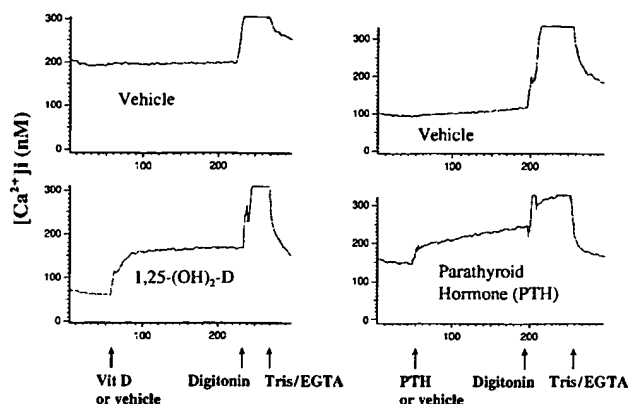
^a Population (mean ± SEM).

Figure 2. Effects of 1,25-(OH)₂-D (left panel) and parathyroid hormone (right panel) on intracellular calcium in human adipocytes.

RESULTS

Figure 2 demonstrates that both 1,25-(OH)₂-D and parathyroid hormone (PTH) stimulate significant, sustained increases in intracellular Ca²⁺ in primary cultures of human adipocytes ($P < 0.001$; EC₅₀ ~ 50 pM for 1,25-(OH)₂-D and ~10 nM for PTH). 1,25-(OH)₂-D treatment also resulted in marked (83%) inhibition of forskolin-stimulated lipolysis ($P < 0.001$) in human adipocytes (Fig. 3). PTH treatment exerted little effect on lipolysis (data not shown) despite its stimulation of an intracellular Ca²⁺ response, most likely as a result of an accompanying activation of adenylate cyclase.

Treatment of aP2-transgenic-agouti mice with the high fat/high sucrose basal diet resulted in a weight gain of 24%, which was reduced by 26 and 29% by the high calcium and medium dairy diets, respectively ($P < 0.04$), and further reduced by 39% by the high dairy diet ($P < 0.04$; Fig. 4). These differences occurred despite the lack of any difference in food intake. Measurement of core temperature, an indirect metabolic index, reflected these observations,

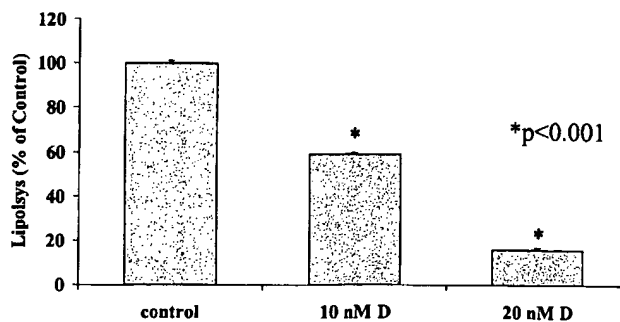


Figure 3. Effects of 1,25-(OH)₂-D on forskolin-stimulated lipolysis in human adipocytes. Forskolin treatment resulted in a ~twofold increase in glycerol release; data shown are normalized to 100% for the forskolin-treated group. (n=6/group; $P < 0.001$).

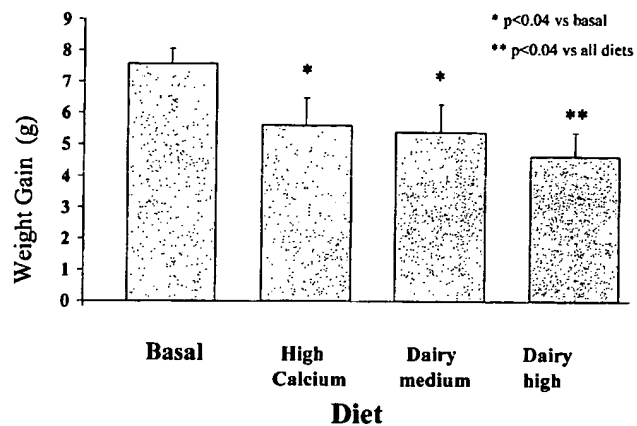


Figure 4. Effects of calcium and dairy products on 6 wk weight gain in transgenic mice expressing *agouti* in adipose tissue under the control of the aP2 promoter.

with $\sim 0.5^{\circ}\text{C}$ increases in core temperature in response to all three high calcium diets ($P<0.03$; Fig. 5). This increase, coupled with the lack of difference in food intake, is indicative of a shift in efficiency of energy metabolism from energy storage to thermogenesis.

This shift in energy metabolism was evident in studies of fatty acid synthase, a key enzyme in *de novo* lipogenesis that is highly sensitive to regulation by nutrients and hormones (12). The basal diet caused a 2.6-fold increase in fatty acid synthase activity, and this effect was markedly attenuated by all three high calcium diets ($P<0.002$; Fig. 6A). The diets caused corresponding decreases in adipocyte fatty acid synthase mRNA, with a 27% reduction on the high calcium diet and a 51% reduction on the medium and high dairy diets ($P<0.01$; Fig. 6B). Adipocyte lipolysis responded to dietary manipulations in an inverse fashion to the fatty acid synthase responses. The basal diet caused a marked (67%) suppression of lipolysis ($P<0.0001$); however, lipolysis was stimulated 3.4- to 5.2-fold by the high calcium diets

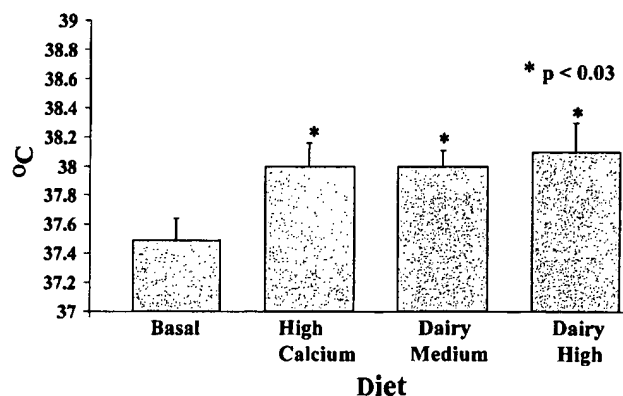


Figure 5. Effects of calcium and dairy products on core temperature in transgenic mice expressing *agouti* in adipose tissue under the control of the aP2 promoter.

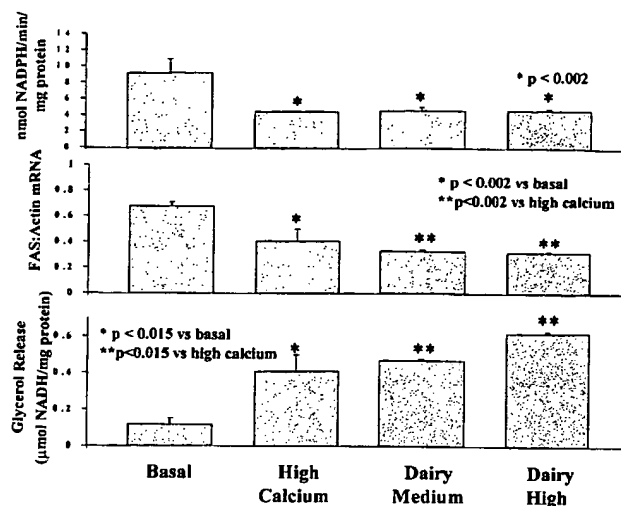


Figure 6. Effects of calcium and dairy products on adipocyte fatty acid synthase activity (upper panel), fatty acid synthase mRNA (middle panel, data are expressed as fatty acid synthase (FAS):actin ratio.) and lipolysis in transgenic mice expressing *agouti* in adipose tissue under the control of the aP2 promoter. Data are expressed as mean \pm standard deviation ($n=10/\text{group}$). Statistical significance is as indicated in each panel.

($P<0.015$; Fig. 6C), with greater effects from the high dairy diets than from the high calcium diet. Assessment of fat pad mass after 6 wk of dietary treatment provides further support for these findings. Table 3 demonstrates that all three high calcium diets caused a 36% reduction in mass of the epididymal, abdominal, perirenal, and subscapular adipose tissue compartments ($P<0.001$). Epididymal and subscapular fat pad mass was reduced by $\sim 50\%$ by all three diets, whereas the abdominal fat pads exhibited greater decreases on the medium and high dairy diets than on the high calcium diet ($P<0.001$; Table 3).

Serial measurements of plasma glucose and insulin demonstrate a diabetogenic effect of the basal high fat/high sucrose/low calcium diet, with an increase in fasting glucose from 98 ± 10 to 130 ± 11 mg/dl ($P<0.02$) and a corresponding degree of compensatory hyperinsulinemia. These increases were attenuated by the high calcium and medium dairy diets and prevented by the high dairy diet (Fig. 7).

Table 4 summarizes the NHANES III data analysis. After controlling for energy intake, activity level, age, race, and ethnicity, the odds ratio of being in the highest quartile of body fat was markedly reduced from 1.00 for the first quartile of calcium intake to 0.75, 0.40, and 0.16 for the second, third, and fourth quartiles, respectively (multiple $R^2=0.20$; $P=0.0009$), in adult women. Similarly, the regression model for males demonstrated an inverse relationship between calcium and dairy intakes and body fat (multiple $R^2=0.40$; $P=0.0006$), although a comparable dose-responsive reduction in relative risk (odds

TABLE 3. Effects of calcium and dairy products on fat pad mass in transgenic mice expressing Agouti in adipose tissue under control of the aP2 promoter^a

	Basal	High calcium	Medium dairy	High dairy
Abdominal (g)	2.239 ± 0.109	1.807 ± 0.082*	1.661 ± 0.127**	1.680 ± 0.113**
Perirenal (g)	1.675 ± 0.124	1.271 ± 0.098*	1.172 ± 0.123*	1.052 ± 0.094*
Epididymal (g)	0.198 ± 0.036	0.110 ± 0.017*	0.110 ± 0.014*	0.097 ± 0.015*
Subscapular (g)	1.592 ± 0.318	0.680 ± 0.069*	0.663 ± 0.068*	0.639 ± 0.087*
Sum ^b (g)	5.703 ± 0.548	3.649 ± 0.238*	3.705 ± 0.276*	3.787 ± 0.251*

^a Data expressed as mean ± standard deviation.

^b Sum of abdominal, perirenal, epididymal, and subscapular fat pads.

* $P < 0.001$ vs. basal. ** $P < 0.001$ vs. high calcium.

ratio) by quartile of calcium intake was not evident from the model.

DISCUSSION

We have previously shown that *agouti*, an obesity gene expressed in human adipocytes, stimulates Ca^{2+} influx (4, 5) and promotes energy storage in human adipocytes by coordinately stimulating the expression and activity of fatty acid synthase, a key enzyme in *de novo* lipogenesis and inhibiting lipolysis in a Ca^{2+} -dependent fashion (6, 7). The carboxyl terminus of agouti protein, which retains functional activity in an *in vitro* assay system (20), bears a striking spatial homology in number and spacing of cysteine residues to spider and snail venoms (ω -conotoxins, plectoxins), which target Ca^{2+} channels (21). Moreover, this agouti modulation of adipocyte lipid metabolism is fully mimicked by Ca^{2+} channel agonists and inhibited by Ca^{2+} channel antagonists (6–8). In addition, treating transgenic mice overexpressing *agouti* with a Ca^{2+} channel antagonist (nifedipine) for 4 wk resulted in significant decreases in

adipocyte lipogenesis and an 18% reduction in adipose tissue mass (9). Thus, adipocyte Ca^{2+} modulates energy storage, suggesting that adipocyte Ca^{2+} is a logical target for pharmacological and/or nutritional regulation of adiposity.

Dietary calcium modulation of intracellular calcium, mediated by suppression of calcitrophic hormones, has previously been demonstrated to attenuate the risk of hypertension, and possibly type II diabetes as well (23, 24). Intracellular calcium plays a key role in multiple related metabolic disorders, including hypertension, cardiac hypertrophy, insulin resistance, and hyperinsulinemia, all of which are commonly associated with obesity. It is now well recognized that these are not merely co-morbid factors that occur secondary to obesity, but rather are part of an integrated metabolic syndrome referred to as 'syndrome X' (25), 'plurimetabolic syndrome', the 'deadly quartet' (obesity, hypertriglyceridemia, hypertension and insulin resistance/hyperinsulinemia), or 'generalized cardiovascular and metabolic disease' (23, 24). Regardless of terminology, a growing body of evidence suggests that these conditions are all characterized by an underlying impairment in intracellular Ca^{2+} (1–3, 23, 24, 26–28). Indeed, Resnick (23) has proposed a unifying 'ionic hypothesis' in which the varying metabolic abnormalities associated with syndrome X represent different tissue-specific manifestations of a cellular lesion characterized, in part, by elevations in steady-state intracellular Ca^{2+} levels. Consistent with this concept, correcting elevations in intracellular Ca^{2+} results in clinical improvements in blood pressure, insulin resistance, platelet aggregation, and left ventricular hypertrophy (23). Our previous studies of the mechanisms of agouti-induced obesity indicate that obesity may also be partly a manifestation of a 'lesion' in intracellular Ca^{2+} regulation (4–7, 9); data from the present study lend further support for this hypothesis.

For adipocyte Ca^{2+} to serve as a logical target for nutritional regulation, human adipocytes would need to exhibit responsiveness to calcitrophic hormones. Our observation in the present study that human adipocytes respond to both parathyroid hormone and

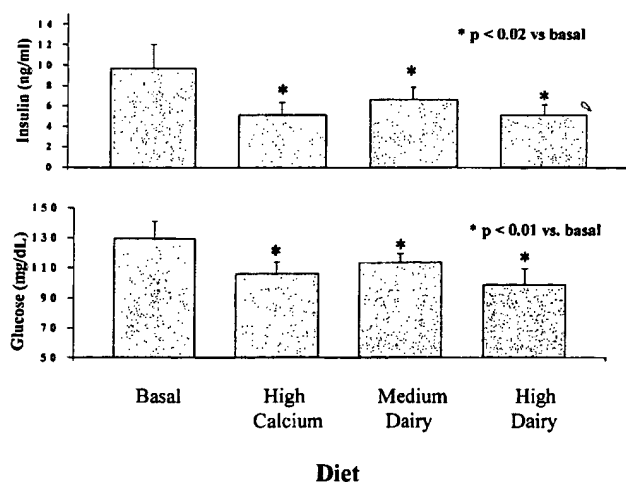


Figure 7. Effects of calcium and dairy products on fasting plasma glucose and insulin levels in transgenic mice expressing *agouti* in adipose tissue under the control of the aP2 promoter. Top panel depicts insulin and the bottom panel depicts glucose.

TABLE 4. Effects of dietary calcium, and dairy intake on the risk of being in the highest quartile of body fat for women^a

Quartile of calcium and dairy consumption	Calcium intake (mg/day; mean \pm SEM)	Dairy consumption (servings/month) mean \pm SEM	Odds ratio of being in the highest body fat quartile
1	255 \pm 20	14.4 \pm 1.9	1.00
2	484 \pm 13	38 \pm 1.3	0.75 (0.13, 4.22) ^b
3	773 \pm 28	57.2 \pm 1.0	0.40 (0.04, 3.90) ^b
4	1346 \pm 113	102.8 \pm 3.6	0.16 (0.03, 0.88) ^b

^a Model is controlled for race/ethnicity and activity level, with age and caloric intake as continuous covariates. ^b 95% Confidence interval in parentheses.

1,25-(OH)₂D with dose-responsive increases in intracellular Ca²⁺ suggests that low Ca²⁺ diets, by virtue of stimulating a calcitrophic hormone response, will increase adipocyte intracellular Ca²⁺ whereas higher calcium diets will suppress this response. Accordingly, a coordinated down-regulation of lipogenesis and up-regulation of lipolysis would be predicted to result from increasing dietary calcium. Data from the present study of transgenic mice overexpressing *agouti* in adipose tissue to mimic the human pattern of expression support this prediction, as the obesity-promoting effects of the lard/sucrose-based diet were significantly attenuated on the high calcium and high dairy diets. These data are further supported by the measurements of fatty acid synthase mRNA and activity, as well as the lipolysis data. Thus, these data demonstrate that increasing dietary calcium attenuates diet-induced adiposity by modulating adipocyte intracellular Ca²⁺ and thereby coordinately regulating lipogenesis and lipolysis (6–9).

Dairy and elemental sources of calcium exerted qualitatively comparable effects; however, calcium in the form of dairy exerted a greater effect on attenuating fat deposition than a comparable quantity of elemental calcium. Consistent with this, a recent randomized clinical trial demonstrated a markedly greater weight loss (7.0 vs. 1.7 kg) in patients maintained on a milk-based diet for 16 wk vs. those maintained on conventional hypocaloric diet at the same level of energy intake (16). Although this difference was attributed to the novelty of the milk-based diet possibly contributing to a greater level of compliance, data presented herein suggest that this effect may also be attributable to suppression of 1,25-(OH)₂D and adipocyte Ca²⁺, with a consequent reduction in the efficiency of energy utilization. This concept is supported by our population-based observations in NHANES III. These data demonstrate a profound reduction in the odds of being in the highest quartile of adiposity associated with increases in calcium and dairy product intake. This analysis was controlled for both energy intake and physical activity. Thus, these data indicate that, for any given level of energy intake and expenditure, a low calcium diet favors increased adipose tissue

energy storage, but the converse was true for higher calcium diets. Accordingly, dietary calcium appears to modulate the efficiency of energy utilization, with low calcium diets favoring increased efficiency of energy storage and higher calcium diets reducing energy efficiency and instead favoring increased thermogenesis. This concept is further supported by our observation of reduced energy efficiency and increased core temperature in the transgenic mice fed the higher calcium diets.

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REFERENCES

- Byyny, R. L., Loverde, M., Llotd, S., Mitchell, W., and Draznin, B. (1992) Cytosolic calcium and insulin resistance in elderly patients with essential hypertension. *Am. J. Hypertens.* **5**, 459–464
- Draznin, B., Sussman, K. E., Eckel, R. H., Kao, M., Yost, T., and Sherman, N. A. (1988) Possible role of cytosolic free calcium concentrations in mediating insulin resistance of obesity and hyperinsulinemia. *J. Clin. Invest.* **82**, 1848–1852
- Draznin, B., Sussman, K., Kao, K., Lewis, D., and Sherman, N. (1987) The existence of an optimal range of cytosolic free calcium for insulin-stimulated glucose transport in rat adipocytes. *J. Biol. Chem.* **262**, 14385–14388
- Kim, J. H., Kiefer, L. L., Woychik, R. P., Wilkison, W. O., Truesdale, A., Ittoop, O., Willard, D. Nichols, J., and Zemel, M. B. (1997) Agouti regulation of intracellular calcium. Role of melanocortin receptor. *Am. J. Physiol.* **272**, E379–E384
- Zemel, M. B., Kim, J. H., Woychik, R. P., Michaud, E. J., Kadwell, S. H., Patel, I. R., and Wilkison, W. O. (1995) Agouti regulation of intracellular calcium: role in the insulin resistance of viable yellow mice. *Proc. Natl. Acad. Sci. USA* **92**, 4733–4737
- Jones, B. H., Kim, J. H., Zemel, M. B., Woychik, R. P., Michaud, E. J., Wilkison, W. O., and Moustaid, N. (1996) Upregulation of adipocyte metabolism by agouti protein: possible paracrine actions in yellow mouse obesity. *Am. J. Physiol.* **270**, E192–E196
- Xue, B. Z., Moustaid, N., Wilkison, W. O., and Zemel, M. B. (1998) The agouti gene product inhibits lipolysis in human adipocytes via a Ca²⁺-dependent mechanism. *FASEB J.* **12**, 1391–1396
- Tebar, F., Soley, M., and Ramirez, I. (1996) The antilipolytic effects of insulin and epidermal growth factor in rat adipocytes are mediated by different mechanisms. *Endocrinology* **137**, 4181–4188
- Kim, J. H., Mynatt, R. L., Moore, J. W., Woychik, R. P., Moustaid, N., and Zemel, M. B. (1996) The effects of calcium channel blockade on agouti induced obesity. *FASEB J.* **10**, 1646–1652
- Mynatt, R. L., Miltenberger, R. J., Klebig, M. L., Zemel, M. B., Wilkinson, J. E., Wilkison, W. O., and Woychik, R. P. (1997) Combined effects of insulin treatment and adipose tissue-

- specific agouti expression on the development of obesity. *Proc. Natl. Acad. Sci. USA* **94**, 919–922; 1997
11. Zemel, M. B., Mynatt, R. L., and Dibling, D. (1999) Synergism between diet-induced hyperinsulinemia and adipocyte-specific agouti expression. *FASEB J.* **13**, 660.3
 12. Moustaid, N., Jones, B. H., and Taylor, J. W. (1996) Insulin increases lipogenic enzyme activity in cultured human adipocytes. *J. Nutr.* **126**, 865–870
 13. National Center for Health Statistics (1994) *Plan and Operation of the Third National Health and Nutrition Examination Survey, 1988–94*, U.S. Government Printing Office, Washington, D.C.
 14. Segal, K. R., Van Loan, M., Fitzgerald, P. I., Hodgdon, J. A., and Van Itallie, T. B. (1988) Lean body mass estimation by bioelectrical impedance: a four-site cross-validation study. *Am. J. Clin. Nutr.* **47**, 7–14
 15. Shah, B. V., Barnwell, B. G., and Bieler, G. S. (1996) *SUDAAN User's Manual, Release 7.0*, Research Triangle Institute, Research Triangle Park, N.C.
 16. Summerbell, C. D., Watts, C., Higgins, J. P. T., and Garrow, J. S. (1998) Randomized controlled trial of novel, simple, and well supervised weight reducing diets in outpatients. *Br. Med. J.* **317**, 487–489
 17. Shi, H., Moustaid-Moussa, N., Wilkison, W. O., and Zemel, M. B. (1999) Role of the sulfonylurea receptor in regulating human adipocyte metabolism. *FASEB J.* In press
 18. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. *J. Biol. Chem.* **260**, 3440–3450
 19. Boobis, L. H., and Manghan, R. J. (1983) A simple one-step enzymatic fluorometric method for the determination of glycerol in 20 μl of plasma. *Clin. Chim. Acta* **132**, 173–179
 20. Willard, D. H., Bodnar, W., Harris, C., Kiefer, L., Nichols, J. S., Blanchard, S., Hoffman, C., Moyer, M., Burkhart, W., Weiel, J., Luther, M. A., Wilkison, W. O., and Rocque, W. J. (1993) Agouti structure and function: characterization of a potent α -melanocyte-stimulating hormone receptor antagonist. *Biochemistry* **34**, 12341–12346
 21. Manne, J., Argeson, A. C., and Siracusa, L. D. (1995) Mechanisms for the pleiotropic effects of the *agouti* gene. *Proc. Natl. Acad. Sci. USA* **92**, 4721–4724
 22. Zemel, M. B., Kim, J. H., Zemel, M. B., Moore, W., Woychik, R. P., Moustaid, N., and Wilkison, W. O. (1995) Agouti gene product regulation of intracellular free calcium results in stimulation of fatty acid synthase. *Obesity Res.* **3**, 338s (abstr.)
 23. Resnick, L. (1999) The cellular ionic basis of hypertension and allied clinical conditions. *Prog. Cardiovasc. Dis.* **42**, 1–22
 24. Zemel, M. B. (1998) Nutritional and endocrine modulation of intracellular calcium: Implications in obesity, insulin resistance and hypertension. *Mol. Cell. Biochem.* **188**, 129–136
 25. Reaven, G. (1988) Role of insulin resistance in human disease. *Diabetes* **37**, 1595–1607
 26. Barbagallo, M., Gupta, R. K., Bardicof, O., Bardicof, M., and Resnick, L. M. (1997) Altered ionic effects of insulin in hypertension: role of basal ion levels in determining cellular responsiveness. *J. Clin. Endocrinol. Metab.* **82**, 1761–1765
 27. Shan, J., Resnick, L. M., Lewanczuk, R. Z., Karpinski, E., Li, B., and Pang, P. K. T. (1993) 1,25-Dihydroxyvitamin D as a cardiovascular hormone: effects on calcium current and cytosolic free calcium in vascular smooth muscle cells. *Am. J. Hypertens.* **6**, 983–988
 28. Resnick, L. M., Gupta, R. K., Bhargava, K. K., Gruenspan, H., Alderman, M. H., and Laragh, J. H. (1991) Cellular ions in hypertension, diabetes and obesity. A nuclear magnetic resonance study. *Hypertension* **17**, 951–957

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